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REPORT DATE: 04/14/2025

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-04-2011		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 APR 2010 - 31 MAR 2011	
4. TITLE AND SUBTITLE  Mechanisms Regulating Plasma Cell Persistence in Health and Autoimmunity				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0185	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  Michael Cancro, Ph.D.  E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  The University of Pennsylvania Philadelphia, PA 19104				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT  We have found that during T-dependent immune responses, nascent plasma cells express RANK ligand as one likely result of cognate interactions that occur in the absence of IL-21. We further show how this upregulation of RANKL may ultimately result in the persistence of long-lived plasma cells.					
15. SUBJECT TERMS long-lived plasma cell generation and persistence; RANKL; plasma cell / myeloid cell interactions; niche induction					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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## Introduction

The TNF (tumor necrosis factor) superfamily of cytokines and receptors was initially found to control apoptosis; however, their roles have been expanded to include multiple aspects of cellular differentiation. One intensively studied TNF subfamily, the B lymphocyte stimulator (BLyS) family of ligands and receptors, controls B cell survival and differentiation. The family includes two cytokines, BLyS and APRIL; and the receptors BR3, TACI, and BCMA (1). BLyS-BR3 interactions mediate primary B cell selection and homeostasis (2). Recent evidence indicates that additional ligand/receptor pairs mediate survival in other functional B cell niches. For example, APRIL promotes the survival of antibody-secreting plasma cells through binding with TACI or BCMA (3).

We have found that long-lived plasma cells generated in T cell dependent antibody responses express another TNF family member, TRANCE, allowing them to induce APRIL production and osteoclastogenesis in bone marrow macrophages. In contrast, short-lived plasma cells generated in T cell independent responses do not express TRANCE. **Our hypothesis is that this bi-directional plasma cell / myeloid cell interaction is key to the establishment of persistent plasma cell pools in the bone marrow and elsewhere.**

We proposed to address this hypothesis through two major aims, each having specific tasks outlined in the Statement of Work and reiterated below. Here, we report on progress made during Year 1, April 2010 through April 2011. Please note that we use the acronym RANKL (receptor activator of NF-kappa B ligand) instead of the older and less descriptive moniker TRANCE (TNF-related activation-induced cytokine), as has become standard practice in the field since our proposal was written.

## Body

In **Aim 1** we will establish whether RANKL-mediated APRIL upregulation and osteoclastogenesis are separable. We also will determine whether RANKL expression is necessary and/or sufficient for establishment of long-lived plasma cell (LLPC) populations.

**Task 1:** Determine whether RANKL-mediated APRIL upregulation in bone marrow macrophages, and subsequent differentiation to osteoclasts, are separable.

**Status:** Accomplished.

**Task 2:** Determine whether RANKL expression is necessary and/or sufficient for establishment of long-lived plasma cell populations. Task 2a is to determine whether RANKL/RANKL-R interactions are important for establishment of LLPC in the bone marrow; and task 2b is to determine whether RANKL expression by



incipient plasma cells, or by helper T cells, is required to establish LLPC pools in bone marrow. Task 2c is to enforce expression of RANKL in B cells responding to antigen, and determine whether a T-independent immune response results in establishment of LLPC pools.

Status: Tasks 2a and 2b have been accomplished; task 2c is scheduled for years 2-3.

Results for Aim 1: Results for Aim 1 include accomplishment of the tasks noted above, along with several additional tasks required to support and extend initial observations.

First, we have shown that only those plasma cells generated in thymus-dependent (TD) immune responses express RANKL (Figure 1, appended manuscript). These are long-lived plasma cells, and they show upregulated RANKL for at least 103 days. In contrast, plasma cells generated during thymus-independent (TI) responses do not express RANKL.

Further, RANKL expression is required for long-lived plasma cell generation and maintenance (Figure 2, appended manuscript). When RANK/RANKL interactions are blocked by administration of RANK-Fc, both total and high-affinity plasma cells are ablated in splenic and bone marrow compartments (Figure 2A). Further, there are significantly fewer plasma cells in mixed chimeras whose B lineage cells are RANKL-deficient (Figure 2B). We observed significant reductions in high-affinity, isotype-switched plasma cells – but not in shorter-lived IgM+ cells – as early as 7 days post-immunization, indicating that RANKL expression in a plasma cell is key to conferring long-lived status.

RANKL expression by TD plasma cells leads to the upregulation of APRIL expression in bone marrow macrophages and osteoclasts (Figure 3, panels A through E). This does not happen when RANK/RANKL interactions are blocked by RANK-Fc, or when the osteoclasts are from APRIL knockout mice (Figure 3F). Moreover, APRIL produced by osteoclasts enhances plasma cell survival (Figure 4). Cell-cell contact is required because APRIL is sequestered on the osteoclast surface. These results also indicate that the differentiation of mature osteoclasts is not necessary for RANKL-induced APRIL production. Therefore, in tissues where osteoclasts are not present, RANKL-expressing plasma cells may induce APRIL production in other myeloid cell types.

Our early results led us to question what might cause RANKL expression to be upregulated in TD plasma cells. In vitro experiments with naïve, pre-immune B cells indicate that one factor is CD40 ligation (Figure 5, appended manuscript). This indicates that cognate interactions, experienced by B cells in during TD responses, are key to RANKL induction - as long as IL-21, a cytokine that reinforces germinal center differentiation, is absent (Figure 5).

Taken together, these results indicate that TD plasma cell-intrinsic expression of RANKL is required for both the generation and maintenance of long-lived plasma

cells. Moreover, in contrast to pre-immune B cell subsets, long-lived plasma cells can apparently induce the generation of their own survival niche within the bone marrow. A manuscript is in preparation (copy appended).

In **Aim 2** we will assess whether the emergence of RANKL-positive, autoantigen-specific plasma cells (PC) is associated with the onset of sustained autoantibody production in the AM14tg mouse model of lupus.

**Task 1:** Determine whether RANKL expression accompanies the emergence of autoantibody-producing plasma cells.

**Approach:** Infuse mice with PL2-3 or control immune complex, harvest spleen and bone marrow, and quantify AM14-producing plasma cells and RANKL expression (Year 1, months 1-6). Sort plasma cells from these experiments for co-culture experiments to determine APRIL production by macrophages.

**Status:** In progress.

**Task 2:** Determine if autoantibody-secreting plasma cells respond to APRIL.

**Approach:** Infuse mice with PL2-3 or control immune complex, harvest spleen and bone marrow, sort plasma cells, and culture in the presence of cytokines, APRIL producing PC or macrophages, with/without inhibitors. Also perform transwell experiments to determine cell contact requirements.

**Status:** In progress.

**Tasks 3 and 4** are scheduled for years 2 and 3. Briefly, these tasks are to determine whether autoantibody-forming cells rely on APRIL or BLyS in vivo; and to determine whether RANKL/RANKL-R interactions are necessary to afford sustained autoantibody production.

## **Key Research Accomplishments**

- We have demonstrated that during T-dependent immune responses, nascent plasma cells are induced to express RANKL, probably by cognate interactions (CD40 ligation) in the absence of the germinal center-promoting cytokine IL-21. This explains, in part, why TD immune responses are effective in generating long-lived plasma cells.
- We have demonstrated one probable mechanism by which long-lived plasma cells result from germinal center reactions. Upregulation of RANKL by nascent plasma cells allows them to interact with macrophages expressing RANK, thereby inducing the macrophages to produce APRIL, which in turn promotes survival of plasma cells for long periods of time.

## Reportable Outcomes

- Manuscript in preparation (draft appended), provisional title: "RANKL expression governs plasma cell persistence"
- Research presentations by the Principal Investigator, M.P. Cancro:

May, 2010 "The Persistent Problems of B Cell Memory." American Society of Gene and Cell Therapy Annual Meeting; Washington, DC

May, 2010 "Commensal Cross-talk between Plasma Cells and Osteoclasts." Kitasato Symposium 2010 on New Prospects for Cytokines; Berlin, Germany

Jun, 2010 "Cellular and Molecular Cross Talk in the Establishment of Humoral Memory." FASEB Summer Conference on The Biology of the Immune System; Carefree, AZ

Jul, 2010 "The Persistence of Memory: Interactions Governing GC Selection and Plasma Cell Survival." Visiting Professor, Annual Research Retreat; Universitat Erlangen, Erlangen, Germany

Sep, 2010 "The Persistence of Memory: Interactions Governing GC Selection and Plasma Cell Survival." Division of Rheumatology Seminar Series; University of Massachusetts School of Medicine, Worcester, MA

Oct, 2010 "Cellular and Molecular Cross-talk in Establishing and Maintaining Humoral Immunity." Immunology Symposium Invited Speaker; University of Washington Immunology Program, Seattle, WA

Jan, 2011 "It Takes Two: Cellular Cross-talk in Germinal Center Initiation and Evolution." Asilomar Midwinter Conference of Immunologists; Asilomar, CA

Jan, 2011 "BLyS: It's Not Just for Primary B Cells Anymore." Stanford University Immunology Program Seminar Series; Palo Alto, CA

Feb, 2011 "The Persistence of Memory: Selection and Survival in Antigen Experienced B Cells." La Jolla Institute for Allergy and Immunology Guest Seminar Series; La Jolla, CA

Apr, 2011 "Plasma Cell Homeostasis and Regulation." American Transplant Congress; Philadelphia, PA

- One Ph.D. obtained with partial support by this award
- Employment opportunities covered in part by this award include one technician, one research scientist, and one postdoctoral trainee

## Conclusions

T cell-dependent immune responses generate long-lived plasma cells, which may persist in an animal for months or years. In contrast, T cell-independent immune responses yield short-lived plasma cells which assist in early defense, but die after a few days or weeks. Our research results provide an explanation of why and how TD immune responses generate long-lived plasma cells. The broader implication is that long-lived, antigen-experienced B cells may induce the generation of their own survival niche, unlike preimmune B cell pools. The central role of RANK/RANKL interactions points to these molecules as potential targets for enhancing antibody production following vaccination; or for amelioration of autoimmune disease.

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## **Appendix**

Draft of manuscript in preparation

# **RANKL expression governs plasma cell persistence**

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## **Abstract**

Plasma cells secrete antibodies associated with both beneficial and adverse immune responses. Two categories of plasma cells, long- and short-lived, are distinguished by their generation requisites, longevity, and anatomic distribution. While thymus dependent immune responses generate long-lived plasma cells most effectively; the underlying mechanism is poorly understood. Here we show that Receptor Activator for Nuclear Factor  $\kappa$  B Ligand (RANKL) is induced in plasma cells generated during thymus-dependent, but not thymus-independent responses. RANKL expression enables plasma cells to interact with myeloid cells, inducing production of APRoliferation Inducing Ligand (APRIL). Disrupting these interactions with RANK-Fc blocks the maintenance of long-lived plasma cells, and RANKL-deficient plasma cells fail to effectively colonize the spleen or bone marrow. Our findings identify plasma cell-intrinsic RANKL expression as indispensable for the generation and maintenance of long-lived plasma cells. Moreover, our data suggest that long-lived plasma cells, unlike preimmune populations, actively instruct the generation of their own survival niche.

## Introduction

Plasma cells are terminally differentiated B lineage cells that secrete immunoglobulins, providing sterilizing immunity to pathogens, as well as mediating both humoral autoimmunity and transplant rejection <sup>1-4</sup>. Following antigen-driven activation, B cells can adopt one of two general plasma cell fates <sup>5-7</sup>. While these can be roughly separated by surface marker expression, cellular lifespan has historically served as the principal means for distinguishing plasma cell pools <sup>6, 8</sup>. So-called short-lived plasma cells primarily inhabit the spleen or lymph nodes, and have an average half-life of about 3 days <sup>8</sup>. In contrast, long-lived plasma cells home mainly to the bone marrow, where they may survive for the life of the individual <sup>6</sup>. Despite considerable information concerning plasma cell generation and survival, fundamental mechanistic questions remain unanswered. The efficient generation of long-lived plasma cells depends on multiple factors, including the nature of the responding B cells <sup>5, 9, 10</sup>, the receipt of cognate T cell help, and the formation of germinal centers <sup>11</sup>. Surprisingly, despite long-standing appreciation that T cell dependent (TD) immune responses most efficiently generate long-lived plasma cells, the underlying mechanisms whereby cognate T help fosters this extraordinary longevity remain obscure. Although several molecular interactions associated with TD responses have been implicated, the lack of downstream, plasma cell intrinsic properties associated with these signals confounds separating their relative roles in germinal center initiation versus long-lived plasma cell differentiation *per se*. For example, while CD40 ligation is important for long-lived plasma cell formation, whether this is simply because long-lived plasma cells must emerge from germinal



centers, or instead indicates that CD40 signals directly drive key events in adopting the long-lived plasma cell fate, is unclear <sup>12</sup>.

Similarly, the survival cytokine requirements of long-lived plasma cells are complex and redundant. Thus, while IL-6 can augment plasma cell survival *in vivo* and *in vitro*, antibody titers are only mildly reduced in IL-6 knockouts<sup>13</sup>, suggesting alternative survival mechanisms. More recently, attention has focused the B Lymphocyte Stimulator (BLyS, also called BAFF {Schneider, 1999 #469}) family cytokines in plasma cell generation and maintenance. This family includes two cytokines, BLyS and A Proliferation Inducing Ligand (APRIL). Both play central roles in B cell homeostasis selection, and differentiation <sup>14</sup>. While BLyS is primarily associated with naïve B cell survival and selection <sup>15</sup>, APRIL plays a substantial role in bone marrow plasma cell homeostasis and IgA responses <sup>16-22</sup>. Indeed, the early bone marrow environment lacks APRIL and thusly cannot support plasma cells <sup>17</sup>. Furthermore, simultaneous elimination of both APRIL and BLyS results in a marked reduction of long-lived plasma cells <sup>18</sup>. Cells of multiple hematopoietic lineages have recently been implicated in APRIL production and plasma cell survival<sup>19, 22</sup>. Furthermore, osteoclasts are a rich source of APRIL and contribute to myeloma growth and survival through a BLyS/APRIL dependent mechanism<sup>23, 24</sup>. Nonetheless, while it is clear that APRIL contributes to plasma cell survival, neither the exact sources of APRIL in each microenvironmental niche, nor the signals required to induce and maintain APRIL expression, are well characterized.

Finally, the chemokine and homing interactions contributing to long-lived plasma cell maintenance, as well as the stromal interactions involved, are poorly understood. Thus, CXCR4 mediated bone marrow homing has been implicated in

the maintenance of long-lived plasma cells <sup>7</sup> prompting models suggesting a bone marrow plasma cell niche of fixed size, in which newly formed plasma cells can only enter the long-lived pool by homing to the BM and supplanting members of previously established cohorts. However, while many long-lived plasma cells reside in the bone marrow, both long-term and transient plasma cell survival niches exist outside of the BM <sup>25</sup>. Accordingly, mechanisms likely exist that allow plasticity in plasma cell numbers, as well as foster the formation and maintenance of extramedullary sites of plasma cell persistence.

In order to probe these remaining questions, we have examined the survival requisites, BLyS receptor profiles, and stromal cell interactions of plasma cells formed in TI and TD responses. Our results show that plasma cells generated in response to TD antigens express RANKL, whereas those generated in TI responses do not. Moreover, in accord with this reliance on cognate T cell help, RANKL expression is induced by CD40 ligation. Finally, we find that RANKL expression enables plasma cells to induce APRIL expression in BM macrophages, licensing them to autonomously induce their survival niche through interactions with RANK expressing myeloid cells. Together, our data provide a molecular mechanism through which TD plasma cells are enabled to autonomously instruct survival niche formation, providing an explanation for the markedly more efficient generation of long lived plasma cells in TD responses.

## Results

### TD immune responses generate RANKL expressing plasma cells

The differing lifespans and anatomic distributions of long- and short-lived plasma cells suggest distinct survival requirements. Accordingly, we compared cytokine receptor expression and responsiveness among bone marrow and splenic plasma cells, focusing on BLyS, APRIL, and IL-6. Splenic plasma cells, which are predominantly short-lived, express the BLyS and APRIL receptor Transmembrane Activator and Calcium-modulator and cyclophilin ligand Interactor (TACI). In contrast, the mainly long-lived bone marrow plasma cell populations exhibit strong B Cell Maturation Antigen (BCMA) expression (Fig. 1 A) <sup>26-28</sup>. Because TACI and BCMA have differing affinities for their ligands, BLyS and APRIL <sup>29</sup>, we tested the abilities of these two cytokines, as well as the established plasma cell survival factor IL-6, to sustain bone marrow or splenic plasma cells *in vitro*. Splenic plasma cells responded well to all three cytokines, whereas at the same cytokine concentrations bone marrow plasma cells preferred APRIL (Supplementary Fig. 1).

### TD immune responses generate RANKL expressing plasma cells

The effectiveness of APRIL in promoting bone marrow plasma cell survival led us to ask whether long-lived plasma cells rely on interactions that foster APRIL production. Because RANKL induces osteoclastogenesis and concomitant APRIL expression in myeloid cells <sup>23, 30-32</sup>, we analyzed RANKL expression during immune responses to either the thymus-dependent antigen, 4-hydroxy-3-nitrophenacetyl conjugated to ovalbumin (NP-OVA); or to the thymus-independent antigen, NP-Ficoll. We examined NP-specific plasma cells in both responses, as well as germinal center B cells and activated CD4 T cells in the thymus-dependent response. As previously reported, activated CD4 T cells expressed RANKL (data

not shown)<sup>33</sup>. Surprisingly, the plasma cells resulting from thymus-dependent immunization, but not germinal center B cells from the same immune response, expressed RANKL. Furthermore, thymus-independent generated plasma cells did not express RANKL (Fig. 1B). This did not reflect sampling time differences, as day 5 thymus-dependent plasma cells also expressed RANKL (Fig. 1C).

### **RANKL interactions are required to sustain plasma cells**

Since thymus-dependent responses engender long-lived plasma cells but thymus-independent responses do not, we hypothesized that RANKL expression might foster longevity by inducing local APRIL production. We reasoned that if RANK-RANKL interactions govern plasma cell persistence, then RANKL-expressing plasma cells should increasingly predominate after thymus-dependent immunization, and that blocking these interactions should deplete existing long-lived plasma cell pools. Consistent with this view, RANKL expression is progressively enriched in long-lived plasma cell pools after immunization (Fig. 1D), such that all bone marrow plasma cells remaining >100 days after thymus-dependent immunization expressed RANKL. To directly test whether RANKL interactions are critical to maintain long-lived plasma cells, we established NP-specific long-lived plasma cells by immunizing mice with 4-hydroxy-3-nitrophenacetyl conjugated to chicken gamma globulin (NP-CGG), then administered 100 $\mu$ g of RANK-Fc or control immunoglobulin at days 52 and 58 post immunization. Subsequent ELISpot analyses revealed that RANK-Fc treatment reduced NP-specific long-lived plasma cells in the spleen and bone marrow by more than 60 and 90 percent, respectively (Fig. 2A).

Having established RANK-RANKL interactions as essential for long-lived plasma cell persistence, we wondered whether RANKL expression by plasma cells themselves is necessary, inasmuch as other cells activated during thymus-

dependent responses also express RANKL<sup>34</sup>. Accordingly, bone marrow chimeras in which RANKL is expressed normally on all cells except the B lineage were made by reconstituting irradiated hosts with mixtures of RANKL-deficient stem cells and RANKL-sufficient, B lineage deficient stem cells. We reasoned that if plasma cell-intrinsic RANKL expression was necessary to establish long-lived plasma cell pools, then these chimeras should have reduced numbers of isotype switched, high affinity plasma cells and bone marrow plasma cells. A significant reduction of isotype-switched splenic plasma cells was evident as early as day 7 post immunization, whereas the early IgM response remained intact (Fig. 2B). Further, total NP-specific plasma cells were significantly reduced in the bone marrow (Fig. 2B), despite normal reconstitution of T and B cells (Supplementary Fig. 3) and normal numbers of GC B cells (not shown). These observations indicate that plasma cell-intrinsic RANKL expression is dispensable for early, short-lived plasma cell generation, but essential to establish high affinity long-lived bone marrow plasma cells.

### **RANKL expressing plasma cells induce APRIL production in BM macrophages**

The requirement for plasma cell-intrinsic RANKL expression led us to hypothesize that incipient plasma cells interact directly with RANK-expressing stromal elements to induce the expression of survival promoting cytokines such as APRIL. In order to test this idea, we co-cultured sorted, NP-specific B cell populations from ongoing thymus-dependent and thymus-independent responses with macrophages for 56 hours (Fig. 3 A-C), then assessed APRIL mRNA levels in the adherent cells (Fig. 3D). Thymus-dependent generated, RANKL<sup>+</sup> plasma cells induced APRIL expression in the adherent cell population, increasing message by >15-fold. Further, this was blocked by RANK-Fc (Fig. 3E). In contrast, neither germinal center B cells from the same thymus-dependent response nor thymus-independent generated plasma cells induced APRIL expression (Fig. 3D). These

data are consistent with the notion that thymus-dependent plasma cells interact with stroma to enable their own survival <sup>17, 21</sup>, a view further supported by experiments where RANKL-induced osteoclasts enabled plasma cell survival in an APRIL-dependent fashion (Fig. 3F).

The need for plasma cell-intrinsic RANKL expression further suggested that cell contact might be required for efficient APRIL capture. Indeed, the osteoclast-mediated support of myeloma cell survival is contact-dependent <sup>35</sup> and can be blocked by TACI-Fc <sup>23</sup>, suggesting that APRIL or BLyS may be sequestered rather than soluble. This is particularly appealing in the case of APRIL, which contains sulfated proteoglycan binding sites. Indeed, while BLyS is readily found in the serum, there is mounting evidence that APRIL is sequestered and acts locally through binding of heparan sulfate proteoglycans <sup>36 37</sup>. We directly assessed whether osteoclasts sequester APRIL by immunofluorescent staining, and found that APRIL decorates the surface of APRIL sufficient, but not APRIL deficient, osteoclasts (Fig. 4A). Moreover, addition of exogenous APRIL to APRIL-deficient osteoclasts led to substantial surface-bound APRIL. Thus, osteoclasts could act as an APRIL depot, necessitating close contact to enable plasma cell survival. To explore this suggestion, we used a Transwell system that physically separates the two cell types, but presents no barrier to passage of soluble factors. Plasma cell survival was enhanced to 75-85% when cultured in the same chamber as osteoclasts, but this enhancement was lost when osteoclasts and plasma cells were cultured in opposite chambers. The requisite for contact was overcome with a high, but not low, concentration of APRIL. This observation could not be explained by an insufficient APRIL concentration or an inefficiency of APRIL to cross the Transwell, since low levels of exogenous APRIL could mediate plasma cell survival when osteoclasts were not present to sequester APRIL (Fig. 4B).

Because osteoclasts are not always present with plasma cells in tissues and because adjacent macrophages make APRIL in extrafollicular plasma cell responses<sup>21</sup>, we asked if the generation of mature osteoclasts per se was necessary for RANKL-induced APRIL production, by culturing bone marrow macrophages with low dose RANKL. We found that macrophages given low dose RANKL induced APRIL production, yet despite becoming lightly Tartrate Resistant Acid Phosphatase positive, the cells retained the size and morphology of a macrophage (Fig. 4C and 4D). This is consistent with the notion that RANKL interactions may be regulating APRIL production in macrophages associated within extrafollicular foci, and may bear on recent findings suggesting APRIL may be induced in a variety of hematopoietic lineages<sup>19, 22</sup>, inasmuch as many of these express RANK.

#### **CD40 induced RANKL expression is blocked by IL-21**

Having established that RANKL expression is a plasma cell intrinsic indicator of the initiation of the long-lived program, we next aimed to determine what aspects of the TD immune response were responsible for plasma cell RANKL expression. To accomplish this, we employed an *in vitro* system to interrogate the signals that lead to RANKL expression among incipient plasma cells. The association of RANKL expression with T-dependent stimulation *in vivo* led us to test whether B cell receptor cross-linking combined with CD40 ligation could induce RANKL transcription. To test this, we cultured 23+ B cells with the indicated cytokines and stimulations for 96 hours, tracking their death and division profiles via TOPRO-3 incorporation and CFSE dilution (Fig. 5A). In fact, compared to B cell receptor cross-linking alone, CD40 ligation induced RANKL expression more than 10 fold (Fig. 5B).

While this indicated that cognate interactions are a key component in RANKL induction and long-lived plasma cell survival, it also raised the conundrum that

germinal center B cells , which similarly require CD40 ligation for their differentiation, do not also express RANKL. Recent findings have suggested that cytokine secretion by T<sub>FH</sub> cells is key to engendering germinal center character, particularly the secretion of IL-21<sup>38-40</sup>. Accordingly, we tested the effects of IL-21 on CD40-mediated RANKL induction. We found that IL-21 was able to repress the induction of RANKL in CD40 stimulated B cells, allowing B cells with the same degree of B cell receptor and CD40 ligation to adopt a distinct RANKL expression profile. The addition of IL-21 was also accompanied by transcriptional changes indicative of germinal center differentiation, including the repression of BLIMP1 and concomitant increase of BCL6 transcripts (Fig. 5B). Together, these data suggest that RANKL expression is driven by CD40 ligation as long as germinal center enforcing cytokines, particularly IL-21, are absent.



## Discussion

These studies have examined the basis for long-lived plasma cell generation and maintenance, focusing on cellular interactions between plasma cells and their surrounding stromal elements. Our results indicate that during TD immune responses, incipient plasma cells are induced to express RANKL by CD40 ligation, but only in the absence of the GC-promoting cytokine IL-21. This observation provides an explanation for the striking effectiveness with which TD immune responses generate long-lived plasma cells, as well as insight regarding the mechanism involved in PC generation from GC B cells. Moreover, we find that RANKL expression enables plasma cells to interact with RANK-expressing macrophages, thereby inducing production of the plasma cell survival cytokine APRIL. Finally, we have shown that PC-intrinsic RANKL expression, as well as RANKL/RANK interactions, are necessary to establish and maintain long-lived plasma cells. Together, these findings prompt a model in which plasma cells derived from TD immune responses, unlike their primary B cell progenitors, are intrinsically licensed to co-opt RANK expressing stromal elements to form survival niches.

Because CD40/CD40L interactions are critical to the generation of both germinal centers and high affinity, class-switched long-lived plasma cells, it is important to understand what these signals contribute to these respective responses. CD40 ligation is capable of mediating a variety of downstream signalling intermediates, culminating in diverse cell fate decisions<sup>12</sup>. Indeed, we find that CD40 ligation alone can increase RANKL and drive a plasma cell program, whereas simultaneous addition of the GC regulatory cytokine, IL-21, extinguishes RANKL expression. This suggests that the GC environment may be restrictive to plasma cell generation or, at minimum, RANKL expression. It further indicates that CD40

ligation in the absence of IL-21 may exclusively promote plasma cell generation. Indeed, it is likely that initial cognate interactions occur prior to the competence of T cells to secrete IL-21, potentiating the early burst of plasma cells seen in TD immune responses<sup>41, 42</sup>. The influence of IL-21 on plasma cell generation also leads to the conundrum of how germinal center cells are able to emerge and initiate a RANKL plasma cell fate despite the presence of IL-21. This could be accomplished several ways, including via restrictions on access or the responsiveness of B cells to IL-21 within the germinal center, or by changing other collaborative signals such as those received through the B cell receptor<sup>43</sup>.

If RANKL expression is sufficient for becoming a long-lived plasma cell, our data suggests that plasma cells capable of seeding this pool are generated very early in a TD immune response. Evidence for the early emergence of long-lived plasma cells has recently been described (Brotnick/Chernova et al. Submitted). This possibility makes the question of selection within the plasma cell pool an intriguing conundrum. For example, if early plasma cells are competent to become long-lived plasma cells then why is the long-lived plasma cell pool predominantly high affinity products of germinal centers? Whether this is because few plasma cells capable of longevity are generated early in a TD response, compared to those generated with a germinal center, or if there is somehow selection within the plasma cell pool per se is unknown.

Recent work from several laboratories has highlighted the importance of APRIL production to the survival of plasma cells<sup>17, 19, 22</sup>. Both megakaryocytes and eosinophils are thought to be important sources of APRIL. Because both of these cells express RANK, it is possible that the unifying feature of APRIL producing cells is RANK expression and that interactions with RANKL expressing cells is what initiates or enhances their APRIL production. This paradigm may also extend to other RANK expressing cells and may prove pathogenic when plasma cell production is augmented, such as in periodontal disease or rheumatoid arthritis.

Our findings *in toto* reveal a reciprocal relationship between plasma cells and their supporting stroma that, combined with evidence that long-lived plasma cells are largely sessile<sup>46, 47</sup>, imply long-lived plasma cells autonomously induce and maintain their own survival habitat. This notion is contrary to prior models of plasma cell survival, which places control of the size and composition of the long-lived plasma cell pool on a pre-established and “fixed” stromal element<sup>7</sup>, whereby plasma cells can only enter into the long-lived pool by supplanting previously established cohorts. Further, if plasma cell survival is dependent on a circumscribed stromal compartment, the survival of plasma cells is reliant on homing, adhesion and a predetermined anatomic locale. We find this model unsatisfying because it neither allows for any elasticity in the number of plasma cells that can be maintained and thus does not account for the increased number of plasma cells found in aged individuals, nor does it allow for a way to protect and maintain plasma cell specificities in the long-lived pool. Further, it does not provide an explanation for how plasma cells can be maintained long-term in inflamed sites and peripheral tissues.

The fact that interactions between plasma cells and RANK expressing cells must be actively maintained without disruption makes RANK/RANKL interactions an

attractive mechanism for maintaining and dissolving inflammatory sites where plasma cells are temporarily found. Disrupting these interactions may also be a valuable target in order to dissolve continuously inflamed, pathogenic plasma cell tissue sites as is found in rheumatoid arthritis and many other plasma cell mediated diseases.

Despite the seeming incongruity of our model with a set anatomic niche, one can imagine areas in which these ideas may overlap. While we assert that RANKL expression is key for plasma cell longevity, there is still a requisite for RANK expressing cells to provide APRIL. While many cells express RANK, it is neither clear that all RANK expressing cells are able to respond to RANKL ligation with APRIL production, nor do we understand the nature and lifespan of the important RANK expressing cells. Because we find that RANK/RANKL interactions must be maintained, we hypothesise that either a static or abundant RANK expressing cell must be available to the plasma cell. This may constitute what other groups consider the static bone marrow niche. Further, this static niche may indeed be enriched for in the bone marrow because of the integral role of RANK/RANKL interactions in maintaining bone homeostasis, thus making the bone marrow a particularly robust anatomic location for plasma cell longevity.

Our data further suggest that the homeostatic mechanisms governing long-lived plasma cell pools differ fundamentally from those active in pre-immune B cell populations. Rather than relying on extrinsically regulated survival factors that circumscribe pool size, the capacity to autonomously instruct survival niche formation allows long-lived plasma cell pools to progressively enlarge with successive immunizations. This enlargement is likely small compared to the total

number of plasma cells generated during a thymus-dependent response suggesting another layer of regulation beyond expression of RANKL. Whether this regulation is intrinsic to the plasma cell or the cells supporting them is unclear. However, it is clear that plasma cell expression of RANKL is a critical component of the mechanism governing plasma cell longevity. Further, the progressive enrichment of long-lived plasma cells for higher RANKL expression makes it tempting to speculate that RANKL expression levels are a key component of that regulation.

The pivotal role played by RANK-RANKL interactions in this process identifies them as possible targets for enhancing long-term antibody production following vaccination. Conversely, the efficiency with which RANK-Fc depletes long-lived plasma cells suggests this may be a useful approach in multiple myeloma, humoral autoimmune syndromes, and illnesses characterized by plasmacytosis and bone erosion.

## Methods

**Mice.** C57BL/6 female mice (age 9-12 wks) were obtained from Jackson Laboratories. BAFF- and APRIL-deficient mice<sup>16, 48</sup> were extensively backcrossed to C57BL/6 and maintained at the University of Southern California (USC). RANKL deficient mice are previously described<sup>49</sup>. The University of Pennsylvania and USC Institutional Animal Care and Use Committees approved all animal procedures.

**Mixed chimeras.** Control chimeras were generated using C57BL/6 bone marrow mixed 3:1 with  $\mu$ MT bone marrow, which are deficient in their ability to generate B cells. To generate chimeras in which B cells alone completely lack RANKL, RANKL deficient fetal liver was harvested at day 14.5 and mixed at 3:1 ratio with  $\mu$ MT bone marrow. One million cells from the appropriate mixtures were then retroorbitally injected into lethally irradiated recipients. All chimeras were allowed to reconstitute for 12 weeks. B and T cell reconstitution was confirmed by examining peripheral blood prior to immunizations.

**Immunizations.** C57BL/6 were immunized intraperitoneally (i.p.) with 100 $\mu$ g NP<sub>16</sub>-CGG in alum, 100 $\mu$ g NP<sub>16</sub>-OVA in alum or 100 $\mu$ g NP<sub>50</sub>-Ficoll in saline.

**Antibodies and FACS.** Splenocytes and bone marrow cells were harvested and stained as previously described<sup>50</sup>. FACS strategies used the following antibodies: biotin-anti-RANKL; PE-Cy5-anti-CD4, anti-CD8 and anti-Gr-1; Alexa700-anti-CD19; Alexa750-anti-B220 (ebioscience). PE-anti-CXCR4, PE-Cy5-anti-F4/80; Biotin-anti-CD3e, anti-GR-1, anti-F4/80 and anti-IgD (BD pharmingen). FITC-anti-Ig $\lambda$ ; PE-anti-Ig $\lambda$  and anti-Ig $\kappa$  (Southern Biotech). Q655-anti-Ig $\kappa$  and APC-NP were conjugated in house. Live cells were identified by pre-incubation with AmCyan fixable live/dead stain (Invitrogen). Cells were fixed and permeabilized using solution A and B (Caltag). Flow cytometry was performed on a BD LSRII. FACS sorting was

performed on a BD ARIA. Analysis was done using FlowJo software (Tree Star, Inc.).

**MACS enrichment.** MACS enrichment was done as previously described<sup>50</sup>.

Depletion of labeled populations was done by collection of the flow through an LD column, while positive selection of CD23 expressing B cells was performed on an LS column (Miltenyi).

**Cytokines and cell culture reagents.** The following reagents were used for cell culture: RANK-Fc<sup>51</sup>; recombinant M-CSF, RANKL, IL-21 and IL-6 (R&D); APRIL (Peprotech); BLyS and TACI-Fc (Human Genome Sciences, Inc.); anti-IgM (Jackson ImmunoResearch); anti-CD40 (BD Bioscience).

**Cell culture.** Macrophages and OC were cultured as described<sup>52</sup>. Briefly, bone marrow cell suspensions were cultured in complete  $\alpha$ -MEM (Gibco) and M-CSF for 3 days. OC were further differentiated by incubation with M-CSF and RANKL in complete  $\alpha$ -MEM for an additional 4 days. OC generation was confirmed by TRAP staining<sup>53</sup>. Sorted plasma cell and antigen specific populations were cultured in complete  $\alpha$ -MEM in the presence of the indicated cytokines, cells or blocking reagents.

**Elispot Assay.** Multiscreen HTS plates (Millipore) were coated with either anti-Ig(H+L) or NP<sub>33</sub>BSA in sodium bicarbonate buffer, then blocked with 2% BSA. Cells were incubated in the plate, undisturbed, for 4-6 hr at 37°C and 5.5% CO<sub>2</sub>. Biotin-anti-Ig $\lambda$  and/or anti-Ig $\kappa$  (Southern Biotech) were added, followed by SA-alkaline phosphatase (Sigma). Spots were detected using BCIP/NBT (Sigma) and scanned and counted on an ImmunoSpot Analyzer (Cellular Technology Ltd.).

## References

**Supplementary Information** accompanies the paper on [www.nature.com/ni](http://www.nature.com/ni).

**Acknowledgements** We thank A. Bhandoola and D. Allman for critical discussions and reading of the manuscript.

**Author Contributions** W.J.Q. performed and analyzed all experiments. T.K. purified RANK-Fc protein and provided expertise. R.G. and P.J.O. performed cell cultures and qPCR. T.M. provided valuable reagents. W.S. and Y.C. provided knockout animals and valuable discussion. W.J.Q. and M.P.C designed the study and wrote the paper.

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## Figure legends

### **Fig 1. Only thymus-dependent plasma cells express RANKL**

(A) Splenic and bone marrow plasma cells were examined for BLyS family receptors fluorocytometrically. Plasma cells were gated as negative for DUMP (a cocktail containing antibodies against GR-1, CD4, CD8, F4/80), sIgM and B220 while being CD138 positive. C57BL/6 animals were immunized i.p. with thymus-dependent (NP-OVA) or thymus-independent (NP-Ficoll) immunogens and splenic NP-binding B cell populations identified at the indicated times.

(B) The peak plasma cell responses to NP-OVA and NP-Ficoll occur at day 7 and day 5 respectively. We therefore harvested spleens from animals at these times post immunization and examined NP<sup>+</sup> populations by surface and intracellular staining. Plasma cells down regulate B220 while germinal center B cells retain both B220 and CD19 and are not found in thymus-independent responses. We further examined these populations for surface expression of RANKL. Gray histogram indicates germinal center B cells, black histograms indicate plasma cells and gray filled histograms are isotype controls. (C) We tested RANKL expression in NP-specific B cell populations by examining thymus-dependent and thymus-independent immunized animals at day 5. Populations were gated as in (B). (D) We tested the kinetics of RANKL expression by thymus-dependent plasma cells at days 7 and 14 in the spleen and days 14 and 103 in the bone marrow. For all panels, data are representative of at least 3 independent experiments. In the experiments shown, n ≥ 3 in all groups.

**Fig. 2. RANKL is requisite for long-lived plasma cell generation and maintenance.**

(A) Mice were immunized with NP-CGG. 100 $\mu$ g RANK-Fc or isotype control was administered i.p. on d52 and d58 post immunization and NP-specific plasma cells determined by elispot at d65. Total NP-specific PC were determined by NP<sub>33</sub>-BSA binding, while high affinity was determined by NP<sub>4</sub>-BSA binding. Data are representative of 3 experiments, each with  $n \geq 3$  in all groups. (B) Mixed bone marrow chimeras were generated as described in methods and immunized with NP-CGG. Plasma cells in the spleen and bone marrow were examined at d7. Significance was determined using two-tailed Data are representative of 2 experiments, each with  $n \geq 4$  in each group. In all panels, significance was assessed by Student T Test. \* =  $p < 0.02$ ; \*\* =  $p < 0.002$ .

**Fig 3. RANKL expressing plasma cells engender APRIL expression**

(A) Mice were immunized with NP-CGG (thymus-dependent) or NP-Ficoll (thymus-independent) antigen and NP-specific B cell populations sorted at day 7 or 5 respectively. (B) mRNA from sorted B cells was reverse transcribed and subjected to qPCR. Expression of various genes relative to GAPDH is shown. Gene levels are normalized to those in thymus-dependent plasma cells. (C) RANKL expression of post sort populations was done flow cytometrically. Filled histograms are isotype control, gray is germinal center B cells and black are plasma cells. (D) In order to determine if NP-specific B cells could induce APRIL production, we co-cultured macrophages with sorted antigen specific populations. RNA from adherent cells after 56 hours was reverse transcribed and subject to qPCR. APRIL expression is shown

relative to GAPDH and normalized to macrophages in M-CSF alone. BLYS transcripts were unchanged between groups (data not shown). (E) We determined the necessity of RANKL-RANK interactions in thymus-dependent plasma cell induction of APRIL by culturing macrophages with soluble RANKL (150ng/ml) or sorted TD plasma cells with or without the addition of RANK-Fc (R-Fc). APRIL expression is shown relative to GAPDH and normalized to macrophages in M-CSF alone. (F) Plasma cells were sorted from bone marrow and plated at a density of  $3 \times 10^3$  cells per well in a 96 well plate with the indicated cytokines and/or osteoclasts generated from indicated genetic backgrounds. TACI-Fc was added to block both BLYS and APRIL. Survival was determined at 24 hours by flow cytometry and Elispot. Data in all panels are representative of at least 3 experiments. Significance was assessed by Student T Test; \*\* =  $p < 0.001$ .

#### **Fig 4. Osteoclasts foster plasma cell survival by production of APRIL**

(A) WT and APRIL knockout osteoclasts were generated and washed thoroughly with medium. APRIL was added as indicated to test the ability of osteoclasts to sequester APRIL. Surface APRIL protein was assessed immunofluorescently. (B) Cell contact is required due to APRIL sequestration by osteoclasts. A Transwell system was used to assess the need for osteoclast/plasma cell contact for survival. Dotted line delineates the upper and lower chamber of the Transwell. Cells and cytokines were added at the indicated doses and sides of the Transwell. Plasma cell survival was assessed at 24 hours post sort via flow cytometry and Elispot. Low RANKL concentration induces APRIL production without mature osteoclastogenesis. Macrophages ( $M\phi$ ) were expanded and cultured with either high or low concentrations of RANKL for 72 hours. Cells were then stained for TRAP (C) or APRIL message tested via RT qPCR (D). Data are representative of 2 to 4

experiments for each co-culture condition, with  $n \geq 3$  in all groups. Significance was assessed by Student T Test  $**=p < 0.0000001$ .

#### Fig 5. CD40 ligation drives RANKL expression in B cells

CD23<sup>+</sup> B cells were magnetically enriched and cultured in medium alone, or in the presence of anti-IgM, anti-CD40 or IL-21 for 96 hours. (A) B cells were labeled with CFSE and after 4 days were analyzed by FACS. TOPRO-3 (100 nM) was added to determine the magnitude of cell death in these cultures. (B) mRNA from cultured B cells was reverse transcribed and subjected to qPCR. Expression of RANKL relative to GAPDH is shown. Gene levels are normalized to B cells stimulated with anti-IgM alone.

#### **Supplementary Fig. 1 Effects of BLyS, APRIL or IL-6 on plasma cell survival *in vitro***

Plasma cell sort strategy and confirmation. Bone marrow was depleted of GR-1, F4/80, CD4, CD8 (DUMP cocktail) and IgD expressing cells via Magnetically Acquired Cell Sorting column. The remaining cells were stained for IgM, B220 and CD138 and FACS sorted based on B220 and IgM negativity, while being CD138 positive. Sorted cells were confirmed as plasma cells by intracellular immunoglobulin expression and lack of surface immunoglobulin. Elispot analysis (300 plasma cells seeded diluted serially left to right 1:3) showed a sort purity of greater than 90%. (B) Plasma cells were sorted from spleen or bone marrow and plated at a density of  $3 \times 10^3$  cells per well in a 96 well plate with either 60ng/ml IL-6, 60ng/ml of BLyS or 100ng/ml APRIL. Survival was determined at 24 hours by flow cytometry and Elispot. Student T Test  $**=p < 0.01$ . Data are representative of  $n > 3$ .

**Supplementary Fig. 2 Confirmation of chimera reconstitution**

Chimera peripheral blood was tested for lymphocyte populations at week 12 post reconstitution. B/T cell ratios as well as immature to mature B cell populations were compared fluorocytometrically. Lymphocyte populations were compared to those found in non-chimeric animals prior to immunization.

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Figure 1

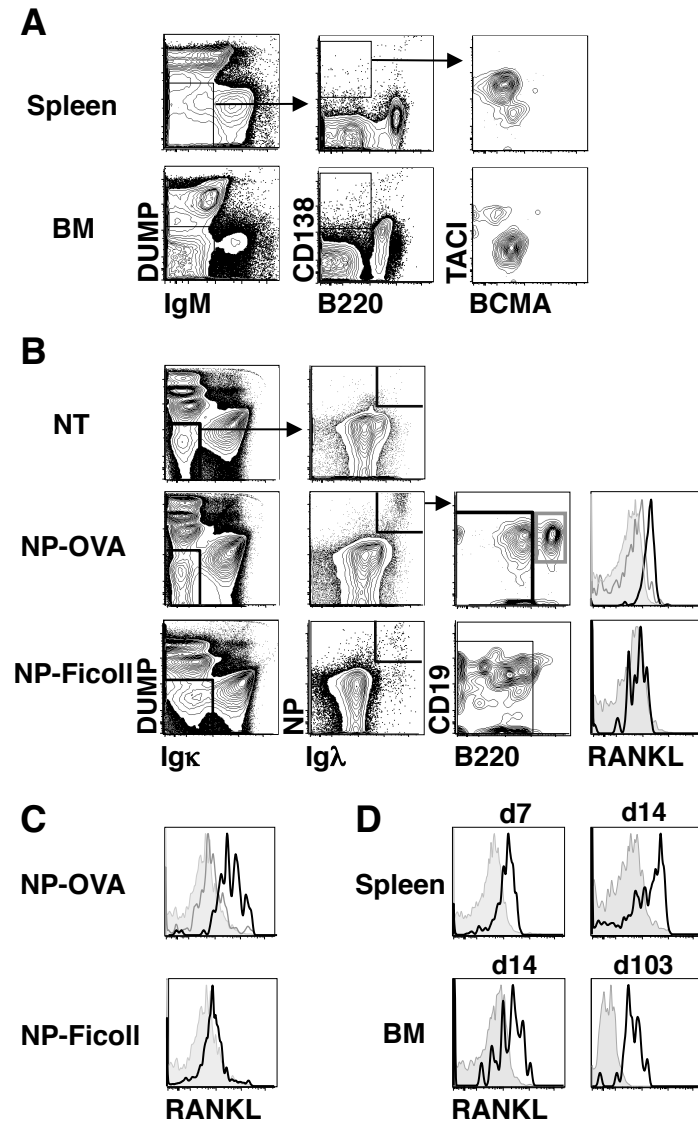


Figure 2

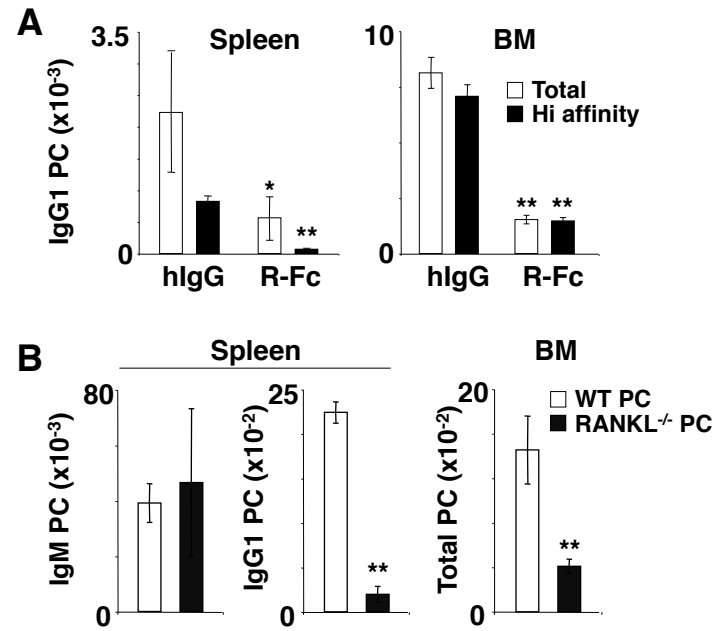


Figure 3

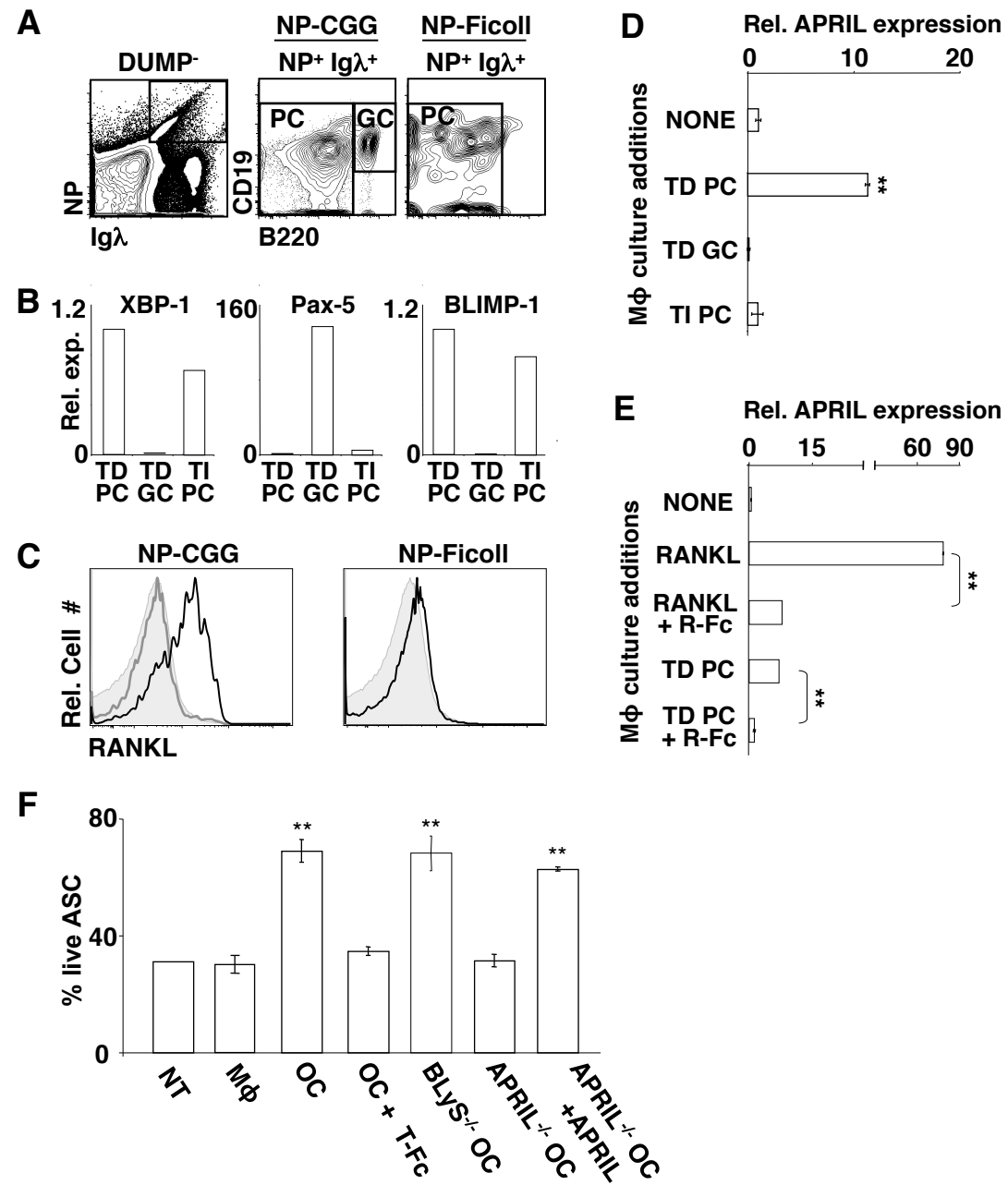


Figure 4

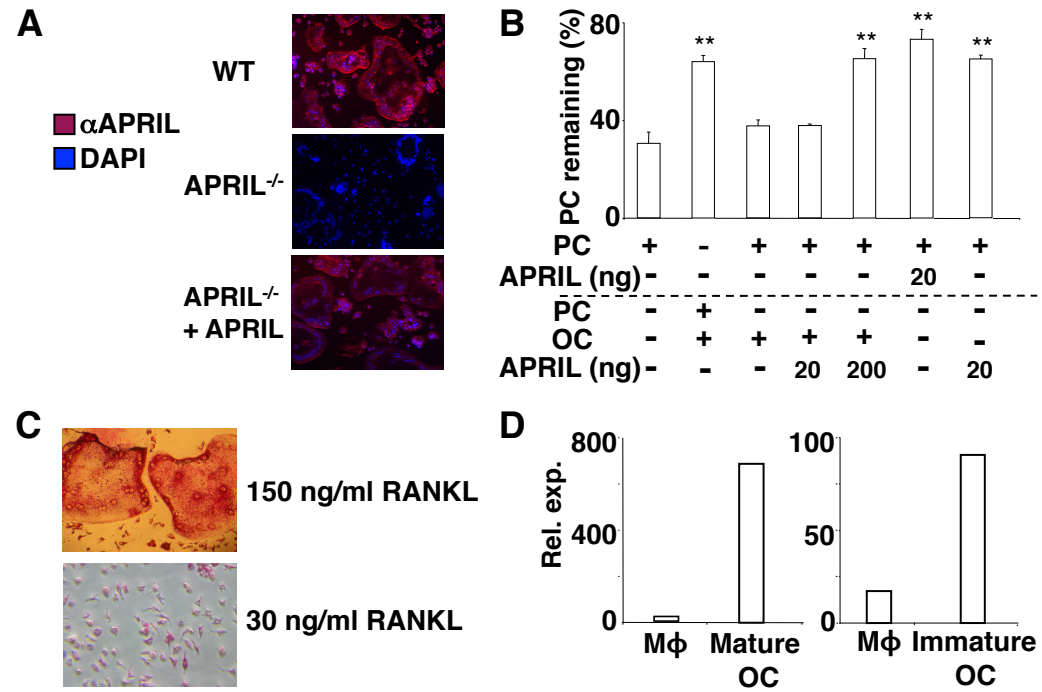
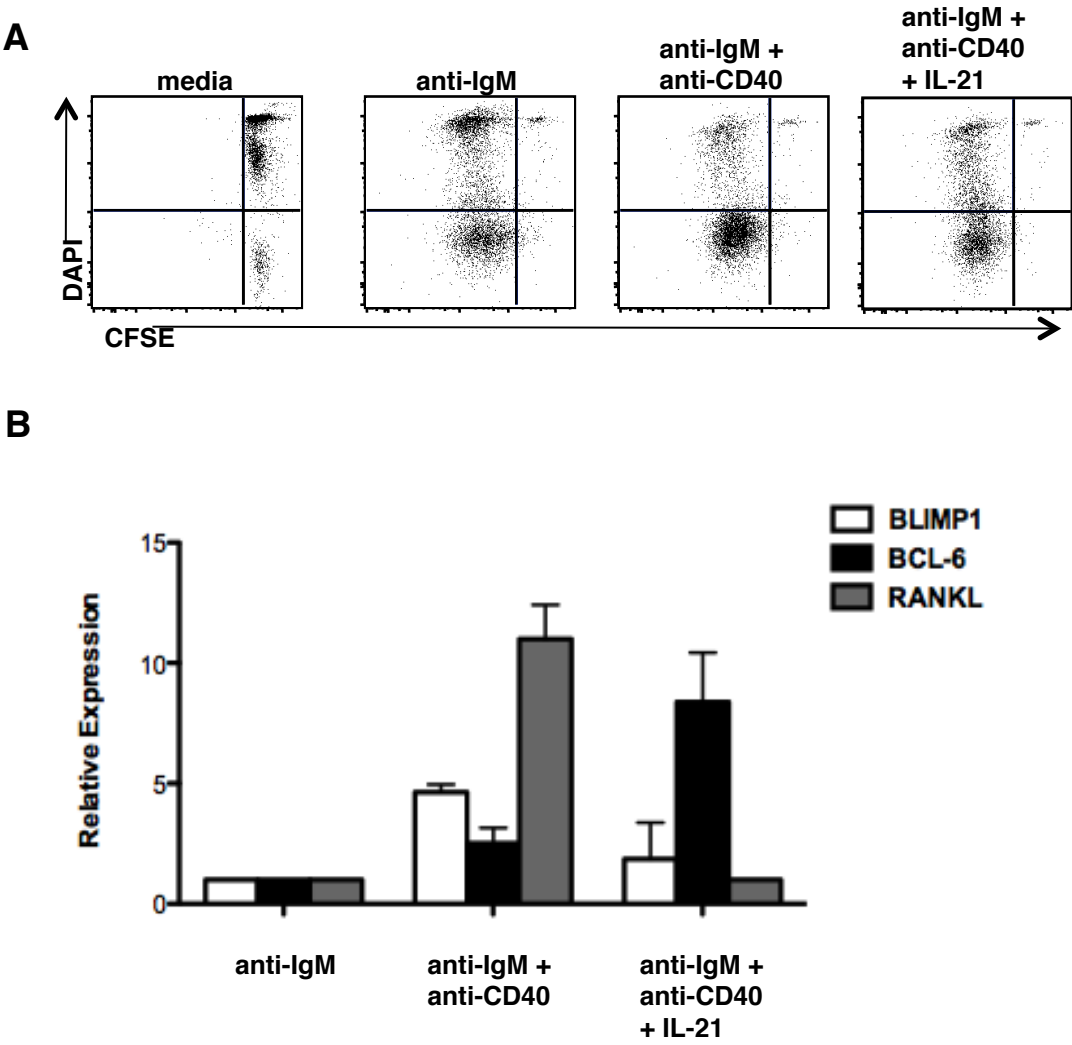
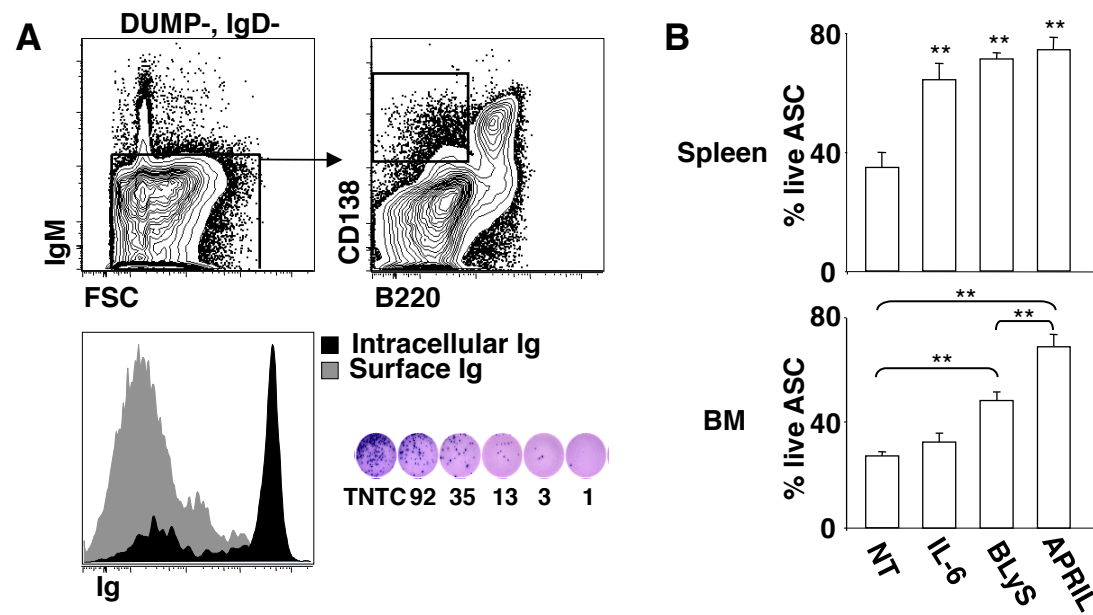


Figure 5



Supplementary Fig. 1



Supplementary Fig. 3

